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REMARKS

Applicants would like to thank the Examiner for the telephonic interview of January 29, 2003, with applicants' attorney, Robert N. Young, in which all of the current rejections in the present case were discussed. During the interview, the Examiner and applicant's attorney came to a general agreement on the substance of claim amendments that would overcome the rejections under 35 U.S.C. §112 and amendments and arguments that may overcome the 35 U.S.C. §103 rejections.

Claims 1-35 are pending in the present application. Claim 1, 11, 17 and 27 have been amended without prejudice in order to advance to the prosecution of the present application. Claims 15, 16, 31 and 32 have been withdrawn as directed to non-elected inventions. Support for the amendments to claims can be found throughout the application as originally filed, including without limitation paragraphs [0014], [0040], [0043] and [0044]. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

I. Claim Rejections – 35 U.S.C. §112

In the Office Action, claims 1, 17 and their dependents were rejected "because the claims fail to recite what 'effects' are being determined[.]" Applicants have amended claims 1 and 17 to point out what constitutes an effect, i.e. "a change in the physical properties or chemical composition of the contacted cells, matrix or tissue relative to a control[,]" as inherently understood in the claim. Applicants note that no control experiment is required to be carried out or a comparison to a control actually be made in order to practice the claimed methods as some effects may be obviously manifested. Rather, were one to compare the test cells, matrix or tissue to a control, the tested cells, matrix or tissue would be different than the control if the test agent(s) had an effect.

The Office Action also stated "it remains unclear what effect, if any, must occur to practice the claimed invention." The claims have been amended to make clear that no effect is

required to occur in order to perform the claimed method as not all test agents will have an effect on the tested cells, matrix or tissue.

Claims 1, 17 and their dependents were also rejected "for reciting both 'tissue engineered cartilage matrix' and 'engineered cartilage tissue' because it is unclear if applicant is using the term interchangeably." The claims have been amended to use consistent terminology throughout the claim which makes clear that the tissue engineered cartilage matrix and the engineered cartilage tissue are not necessarily coextensive, although there may be significant overlap between the two.

Claims 1, 17 and their dependents were also rejected as "indefinite because while the method is drawn to determining effects on an engineered cartilage tissue (ECT), step (B) allows for the determining step to occur on isolated chondrogenic cells before they have formed into the ECT. It is unclear how one could determine the effects of an agent on an ECT by contacting cells that have yet to form an ECT." The claims have been amended to use consistent terminology throughout and specify that any effect of the test agent can be manifested on any of the contacted, cells, matrices or tissues.

Claims 11 and 27 were again rejected "because it is unclear if step (C) rather comprises enzymatically degrading the ECT, or further comprises enzymatically degrading ECT." Claims 11 and 27 have been amended to specify that (C) further comprises enzymatically degrading the engineered cartilage tissue obviating the ground for this rejection. As such, the first instance cited in the Office Action, where "step (C) rather comprises enzymatically degrading the ECT" is fully encompassed by other claims.

Because the claims "apprise[] one of ordinary skill in the art of [their] scope and, therefore, serve[] the notice function required by 35 U.S.C. §112, second paragraph" applicants respectfully request the Examiner withdraw these rejections. MPEP §2173.02.

II. Claim Rejections – 35 U.S.C. §103

In the Office Action, claims 1-8, 10, 14, 17-24, 26, 29-30 and 33 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Kai *et al.* (JP 2001 089390 A) in view of Masuda *et al.* (US 6197061 B1)." Applicants respectfully traverse this rejection. In order to establish a *prima facie* case of obviousness "the prior art reference (or references when combined) must teach or suggest all the claim limitations." However, the combination of Kai *et al.* and Masuda *et al.* cannot state a proper *prima facie* case of obviousness because the references alone or in combination fail to disclose or suggest an engineered cartilage matrix that can be rapidly degraded. Such a rapid matrix turnover is advantageous as "this rapid degradation lends itself to high throughput screening methods because testing of compounds can be completed in a relatively short amount of time" and "characterization of the cartilage matrix, and thus the effect of test agents thereon, is simplified." Present application, paragraphs [0044] and [0056], respectively. Neither of the references teach or suggest these advantages. Applicants enclose herewith a copy of Aydelotte *et al.*, Articular Cartilage and Osteoarthritis 237-49 (1992), which disclose that the turnover rate of typical chondrocyte cell cultures is much slower than presently claimed. See, for example, FIG. 2., As the cited references fail to teach or suggest all of the elements of the presently claims methods, applicants respectfully request the Examiner withdraw this rejection.

Claims 1-10, 14, 17-26, 33 and 35 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Purchio *et al.* (US 5902741) in view of Masuda." As above, these references, taken alone or in combination, cannot state a proper *prima facie* case of obviousness because they fail to disclose or suggest all of the elements of the present claims, namely that the cartilage matrix can be rapidly degraded. Therefore, applicants respectfully request the Examiner withdraw this rejection.

Claim 1-8, 17-24, 29-30 and 35 were also "rejected under 35 U.S.C. 103(a) as being unpatentable over Saito (July 1999) in view of Masuda *et al.*" Similar to above, these references taken alone or in combination fail to disclose or suggest a rapidly degradable cartilage

matrix as presently claimed. Accordingly, the references cannot state a proper *prima facie* case of obviousness and applicants respectfully request the Examiner withdraw the rejection.

Claims 1-11, 17-27 and 29-30 were also "rejected under 35 U.S.C. 103(a) as being unpatentable over Huch *et al.* (1997) in view of Masuda." This rejection must also fail for the reasons discussed above because the references do not disclose or suggest a rapidly degradable cartilage matrix or the advantages that can be achieved therefrom.

Finally, claims 1-8, 10, 14, 17-24, 26 and 33 were "rejected under 35 U.S.C. 103(a) as being unpatentable over Lansbury *et al.* (WO 94/28889) in view of Masuda." This combination also does not state a *prima facie* case of obviousness for the reasons discussed above, as the references do not disclose or suggest a cartilage matrix that can be rapidly degraded. Therefore, applicants also respectfully request the Examiner withdraw this rejection.

CONCLUSION

In view of the above remarks, it is respectfully submitted that this application is in condition for allowance. Early notice to that effect is earnestly solicited. The Examiner is invited to telephone the undersigned at the number listed below if the Examiner believes such would be helpful in advancing the application to issue.

Respectfully submitted,

Date February 17, 2004 By _____

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Articular Cartilage and Osteoarthritis,
edited by K. Kuettner et al.
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Heterogeneity of Articular Chondrocytes

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The structure of articular cartilage has been described in detail in earlier chapters of this book (see chapters by Hunziker and by Poole); further elaboration is not necessary here except in relation to the zonal variation in depth, which provides the most striking heterogeneity in articular cartilage and is the focus of the present discussion. Four layers are generally recognized from the articular surface to the subchondral bone, namely the superficial (tangential), middle (transitional), deep (radial), and calcified zones (I–IV, respectively). These subdivisions are based on well-recognized morphological differences in (a) cell density and orientation, (b) nature, content, and distribution of proteoglycans, and (c) organization of the collagenous fibrillar network, which all vary with distance from the articular surface (for a recent review see ref. 1). The material properties of the cartilage at different depths also change because they are determined by the biochemical nature, content, and organization of the matrix macromolecules.

Because the living chondrocytes elaborate and maintain this complex matrix in which they reside, the zonal variations in the extracellular matrix must result from metabolic differences among the cells. However, it is not known to what degree the metabolism of articular chondrocytes varies because of intrinsic cellular specializations that arise during growth and differentiation, or because of modulation in response to the cells' environment within the tissue. Several investigators have recently addressed this question by examining the morphology and metabolism of subpopulations of cultured articular chondrocytes following their isolation from different depths of the cartilage (2–7). Results show striking differences between chondrocytes derived from the superficial and the deep slices of cartilage in terms of their morphology, metabolism, phenotypic stability, and responsiveness to interleukin-1α (IL-1). Zonal differences in metabolism have also been demonstrated in cartilage explant cultures when chondrocytes are left undisturbed within their original matrix (8,9). Such studies may provide one way to understand better how these diverse cell populations contribute to the long-term maintenance of healthy articular cartilage.

MORPHOLOGY OF CULTURED CHONDROCYTES

Current methods of dissecting articular cartilage to separate groups of cells have not yielded pure preparations of different types of chondrocytes, because the slices do not correspond precisely with the different zones of the tissue. Furthermore, changes in the tissue with depth are gradual, not abrupt, and even within one zone there may be considerable cellular heterogeneity. These considerations must be kept in mind when studies of chondrocytes derived from superficial, middle, and deep regions of cartilage (i.e., cells from zones I, II, and III) are described. Nevertheless, the cell populations from these individual zones are certainly more homogeneous than are preparations isolated from the entire thickness of the tissue, and they show marked morphological and metabolic differences in culture. There is generally good agreement in results with these populations of chondrocytes from bovine, porcine, and human articular cartilage (4,6,7). The middle slices of cartilage which are harvested include some residual tangential tissue, most of the transitional zone, and the upper part of the radial zone; these slices yield a heterogeneous population of chondrocytes which always gives results that are similar to those of the full population and that are intermediate between populations from superficial and deep slices. In intact tissue, this region has the highest concentration of proteoglycans, and it is frequently the source of cells which proliferate to form cell nests in damaged osteoarthritic cartilage.

Chondrocytic metabolism is readily modulated by alterations in cell shape (10) or by changes in the organization of cytoskeletal microfilaments (11). Thus, the results of experiments with isolated cells depend to some extent on the environmental conditions provided by the particular culture method. For example, differences exhibited initially between the populations of cells derived from superficial and deep zones of cartilage diminished with time in monolayer culture at low density under conditions in which the chondrocytic phenotype is labile (6,7). However, in high-density monolayers, and especially in suspension culture either within an agarose gel or in liquid medium over agarose, some phenotypic differences between chondrocytes from zones I and III were retained for extended periods (4,6,7). Chondrocytes from zone I, cultured in agarose gel, became irregular in shape, with numerous processes (3,4), and in liquid medium they formed clusters covered by flattened cells resembling a perichondrium (7). Some of the cells from zone I produced very little extracellular matrix, whereas others became surrounded by a highly fibrillar matrix poor in proteoglycans (4,7). In contrast, chondrocytes from the deep zone retained a rounded shape and morphological features typical for chondrocytes. These cells, cultured within an agarose gel, accumulated an extensive extracellular matrix rich in proteoglycans and containing collagen fibrils (4,7). Similarly, in liquid medium the clusters of rounded chondrocytes from the deep zone produced large quantities of matrix that contained proteoglycan granules and fine collagen fibrils, and they lacked a covering of flattened cells (7). The matrix which is formed in these agarose cultures is denser and more highly organized than that formed in liquid medium,

presumably because the agarose is more effective in trapping the matrix macromolecules close to the cells.

CELL PROLIFERATION IN CULTURE

Although there is little, if any, cell division in healthy adult articular cartilage, chondrocytes cultured in medium containing fetal bovine serum proliferated in response to serum growth factors. The doubling time for bovine chondrocytes

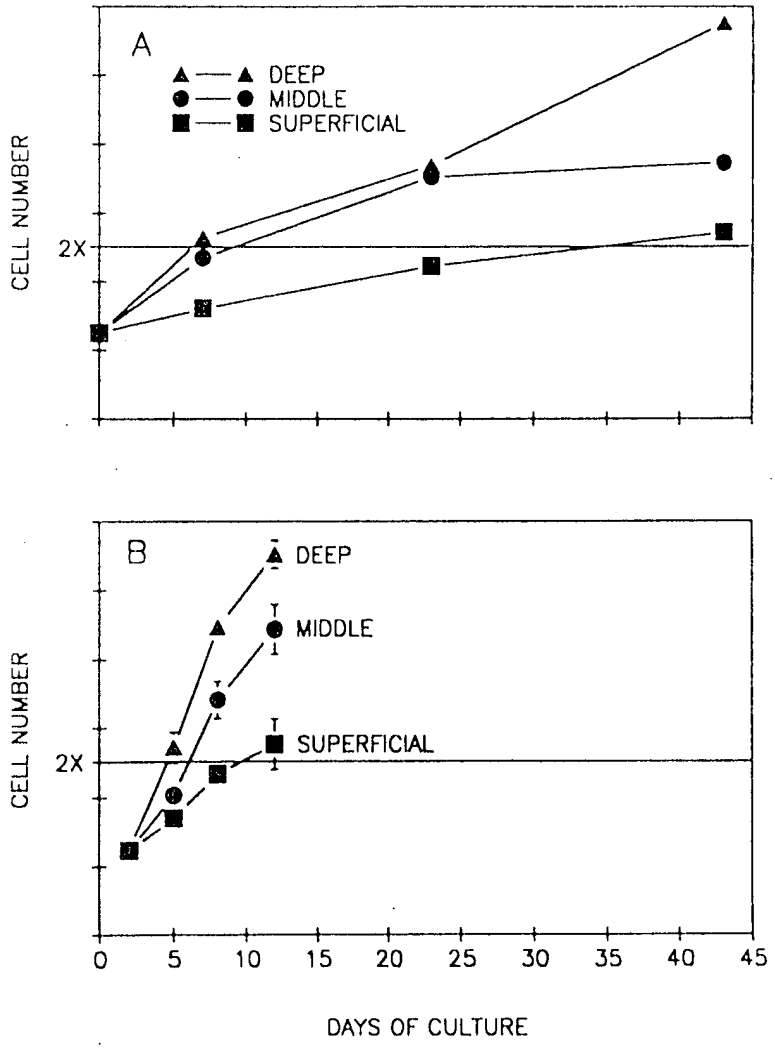

FIG. 1. Growth of subpopulations of bovine chondrocytes derived from superficial, middle, and deep regions of articular cartilage after different times of incubation within an agarose gel. **A:** At high density, mean cell numbers were calculated from measurements of DNA in duplicate samples using the fluorescent Hoechst dye 33258. **B:** Cells cultured at very low density (1/32 that for **A**) were counted under the microscope in defined areas on days indicated (vertical bars, visible only where larger than symbols, show standard deviations of the mean of three or four determinations). Note that proliferation was more rapid in low-density than in high-density cultures, but that under both conditions, chondrocytes from the deep zone of cartilage divided more rapidly than those from the middle region, and cells from the superficial zone showed the least proliferation. Horizontal lines at 2X mark doubling of the populations.

varied both with the cell density and with the source of cells within the articular cartilage (Fig. 1). Proliferation was more rapid in low-density than in high-density cultures, in agreement with results for porcine chondrocytes (6); however, at both high and low densities in agarose gels, chondrocytes from zone III divided more rapidly than did those from zone I. The doubling times for zone III chondrocytes at low and high density were approximately 5 days and 7 days, respectively, compared with 9 days and 32 days for the zone I cells. At high density, porcine chondrocytes from deep layers of cartilage reached greater numbers at confluence than did cells from superficial layers; this difference can be explained partly by the more flattened shape of cells from the upper zone (6). Subpopulations of human articular chondrocytes maintained in medium containing adult human serum, which has lower concentrations of growth factors than does fetal serum, showed little change in cell number during the culture period, and no difference in proliferation was reported between cells from the superficial and deep zones (12).

PROTEOGLYCAN SYNTHESIS

Chondrocytes derived from the proteoglycan-rich matrix of zone III consistently synthesized more proteoglycans than did cells from zone I, which normally reside in a matrix with a relatively low concentration of proteoglycans (4–7). Chondrocytes from both the superficial and deep zones of articular cartilage synthesized predominantly the proteoglycan, aggrecan, but zone I bovine and porcine cells produced relatively more of the small, nonaggregating proteoglycans than did zone III cells (5,6). In suspension cultures of human chondrocytes from both the superficial and deep regions of cartilage, aggrecan accounted for approximately 85% of the total ^{35}S incorporated into proteoglycans, although in the early stages of culture most cells were shown by immunohistochemistry to be positive for the dermatan sulfate proteoglycan, decorin (12). When grown as monolayers, in addition to aggrecan, human chondrocytes from both zones I and III also synthesized significant amounts of smaller, nonaggregating proteoglycans at later stages of culture (7). The quantitative differences in metabolism of proteoglycans observed in isolated chondrocytes cultured in suspension were retained throughout the culture period, and are in keeping with variations in the biochemical composition of matrix which surrounds these cells *in vivo.*

Keratan sulfate was synthesized during early stages of culture by most of the chondrocytes derived from zone III, but by only a few of those from the superficial zone, whether they were grown as monolayers or in suspension (2,6,7,13). Furthermore, keratan sulfate accounted for a higher percentage of ^{35}S in proteoglycans synthesized by zone III than by zone I chondrocytes (6,13). This metabolic difference coincides with the higher content of keratan sulfate in proteoglycans deep within the tissue than in those near the articular surface. With extended time *in vitro,* most of the zone I cells from porcine and human cartilage also

began to synthesize keratan sulfate, but the reason for this modulation is not clear (2,7).

COLLAGEN SYNTHESIS

Collagen production by subpopulations of bovine articular chondrocytes has been examined in collaboration with Dr. Thomas Schmid, for periods of up to 6 weeks using [^{3}H]proline as a precursor (14). Type II collagen was the predominant form synthesized by all populations, and at all stages; no type I collagen was detectable after 1 week, although small quantities were synthesized at later stages of culture after cells had formed a monolayer underneath or on the surface of the agarose. Chondrocytes of zone III were the most active in collagen production; after 1 week, the proportions of collagen synthesized by chondrocytes from superficial, middle, and deep zones were approximately 1:2:3, respectively. These marked quantitative differences in collagen synthesis remained essentially unchanged after 3 and 6 weeks, although the net synthetic rate declined steadily with time in culture (14). Interestingly, small quantities of type X collagen were detected after 6 weeks in cultured bovine chondrocytes from zone III (T. Schmid, *personal communication*), but human chondrocytes from all zones initiated synthesis of type X collagen in liquid suspension culture (15).

Results of immunohistochemical staining with antibodies directed against collagens were in keeping with the more active synthesis of collagen by chondrocytes from zone III than by those from zone I. The percentage of cells with matrix binding an antibody for type II collagen (anti-chick type II collagen from Dr. T. Linsenmeyer) was much higher (and the staining was more intense) in the subpopulation from the deep zone than in that from the superficial zone. Extracellular matrix rich in proteoglycans which stained with alcian blue also stained for type II collagen. After 3 weeks in liquid suspension culture, over 90% of the chondrocytes from superficial and deep zones of human cartilage produced type II collagen detectable by immunofluorescence (7). Collagen of types IX and XI were also synthesized by bovine chondrocytes (detected by immunohistochemistry, using antibodies against bovine collagens, kindly provided by Dr. D. Hartmann), and their distribution was essentially similar to that of type II collagen.

RESPONSE TO INTERLEUKIN-1

Another important metabolic difference between chondrocytes derived from superficial and deep zones of the cartilage lies in their responsiveness to IL-1. Studies of cultured cartilage slices or the entire population of chondrocytes have demonstrated that this cytokine has a dual action. It inhibits synthesis of proteoglycans and also stimulates production of chondrocytic proteases (such as stromelysin, collagenase, and gelatinase), which results in enhanced catabolism

of matrix—that is, chondrocytic chondrolysis (e.g., see refs. 16–19; also see chapter by Tyler et al.). Human articular chondrocytes cultured in either agarose gel or alginate beads also responded to IL-1 with diminished proteoglycan synthesis, but little catabolic response was observed (see chapter by Häuselmann et al., *this volume*). It is well accepted that IL-1 plays an important role in the pathology of inflammatory joint diseases, such as rheumatoid arthritis, but it may also contribute to cartilage damage during inflammatory episodes in osteoarthritis by stimulating matrix degradation and inhibiting repair. Therefore, it is important to understand how this cytokine influences chondrocytes and their surrounding matrix.

Bovine chondrocytes derived from zone I have consistently shown a greater sensitivity to IL-1α than have chondrocytes from the deep zone, based upon both their catabolic and anabolic responses (20). In control medium, chondrocytes from the superficial zone degraded their newly synthesized proteoglycans more rapidly than did zone III chondrocytes, but catabolism was greatly enhanced in the presence of IL-1 (Fig. 2). Zone III chondrocytes, on the other hand, showed only slightly accelerated catabolism at the same concentration of IL-1 (Fig. 2).

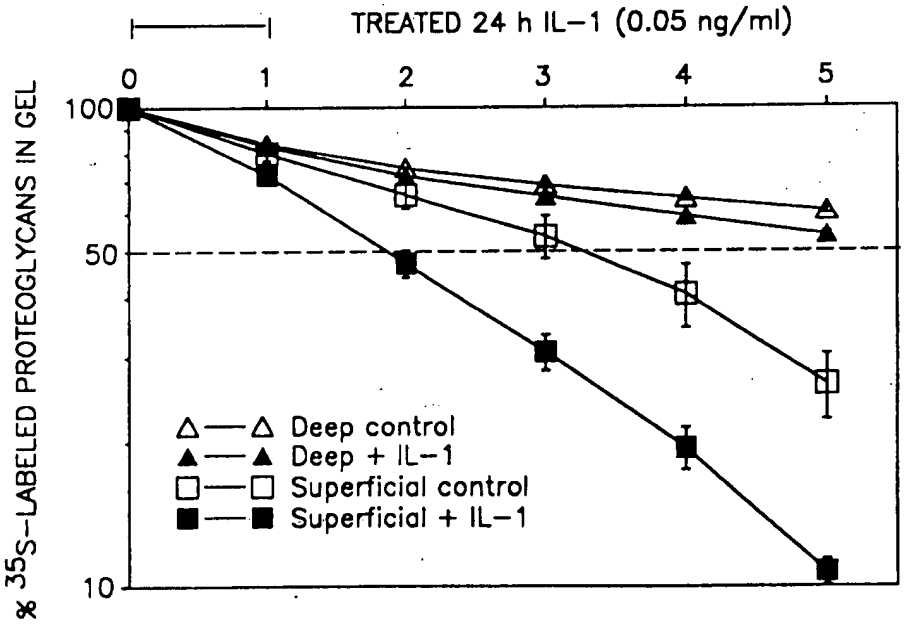

FIG. 2. Graph showing the percentage of ^{35}S-labeled proteoglycans remaining in the agarose-gel–cell layer on different days for control cultures (*open symbols*) and cultures treated for 24 hr with IL-1 (0.05 ng/ml) (*closed symbols*). After 4 days in control medium, cultures were labeled for 16 hr with [^{35}S]sulfate, then rinsed repeatedly to remove remaining free isotope, and the medium was changed and harvested daily for the remainder of the experiment. IL-1 was added to one group of cultures of superficial- and deep-zone chondrocytes for 24 hr. At the end of the experiment, the macromolecular radioactivity in harvested media and in the agarose-gel–cell layer was measured by liquid scintillation spectroscopy. Note that control cultures of chondrocytes derived from deep cartilage (*triangles*) retained a much greater percentage of proteoglycans than did cultures of cells from superficial cartilage (*squares*). In addition, IL-1 elicited a greater catabolic response in the population of superficial-zone chondrocytes, resulting in an additional loss of 16% ^{35}S-labeled proteoglycans, compared with only 7% for the deep-zone chondrocytes.

In terms of long-term damage to cartilage matrix by IL-1, the inhibition of proteoglycan synthesis may play a more important role than does the stimulation of matrix catabolism. In this context, it is interesting that the concentration of IL-1 required for 50% inhibition of proteoglycan synthesis for chondrocytes from zone III was approximately 25-fold that which elicited a similar response from cells of zone I. Further studies are needed to understand the basis for this cellular difference. The number of cellular receptors for IL-1 on the chondrocytes derived from superficial and deep cartilage may differ, the distribution of IL-1 in the vicinity of the cells may be affected by differences in the nature of their surrounding extracellular matrix, and subpopulations of chondrocytes may differ in their capacity for synthesis of endogenous IL-1 (21). However, these results with IL-1 on cultured subpopulations suggest that in intact articular cartilage, the chondrocytes which would be the most responsive to IL-1 *in vitro* are those which are close to the surface of the tissue, and most likely to be exposed to IL-1 in synovial fluid.

LONG-TERM CULTURES IN AGAROSE GEL

The different capacities of chondrocytes from zones I and III to accumulate an extracellular matrix when grown under similar conditions in agarose gel were obvious at early stages of culture, but became progressively more marked with time. The higher rates of synthesis of proteoglycans and collagens contribute to these differences, as does the greater retention of these macromolecules in the vicinity of zone III chondrocytes (4,5). Although some zone I cells produced a fibrillar matrix poor in proteoglycans, others retained very little morphologically distinct matrix. Even in a mixed population of chondrocytes from zone II, after an initial accumulation of compact matrix around some of the cells, this matrix was gradually lost at later stages of culture, coincident with an overgrowth of irregularly shaped cells which lacked matrix (Fig. 3A). Conversely, during a period of 2–6 weeks, chondrocytes from zone III surrounded themselves with a highly organized extracellular matrix and formed chondron-like structures (Fig. 3B). Such matrix consisted of both a proteoglycan-rich pericellular zone and a more peripheral layer resembling a territorial matrix, with collagen fibers oriented primarily parallel to the cell surface (Fig. 4). This matrix showed strong birefringence when examined by polarizing microscopy, as a result of the circular orientation of the collagen fibers (Fig. 5) (22). Chondrocytes from deep layers of cartilage which elaborated this kind of matrix were stable in culture for long periods and could be maintained for over 6 months with little change except for growing cell-clusters near the periphery of the culture. In our experience, zone I chondrocytes have not assembled a similar cartilaginous matrix nor formed stable populations when cultured within agarose.

Many chondrocytes from deep layers of cartilage, but not those from other zones, enlarged and became hypertrophic after several weeks. Changes charac-

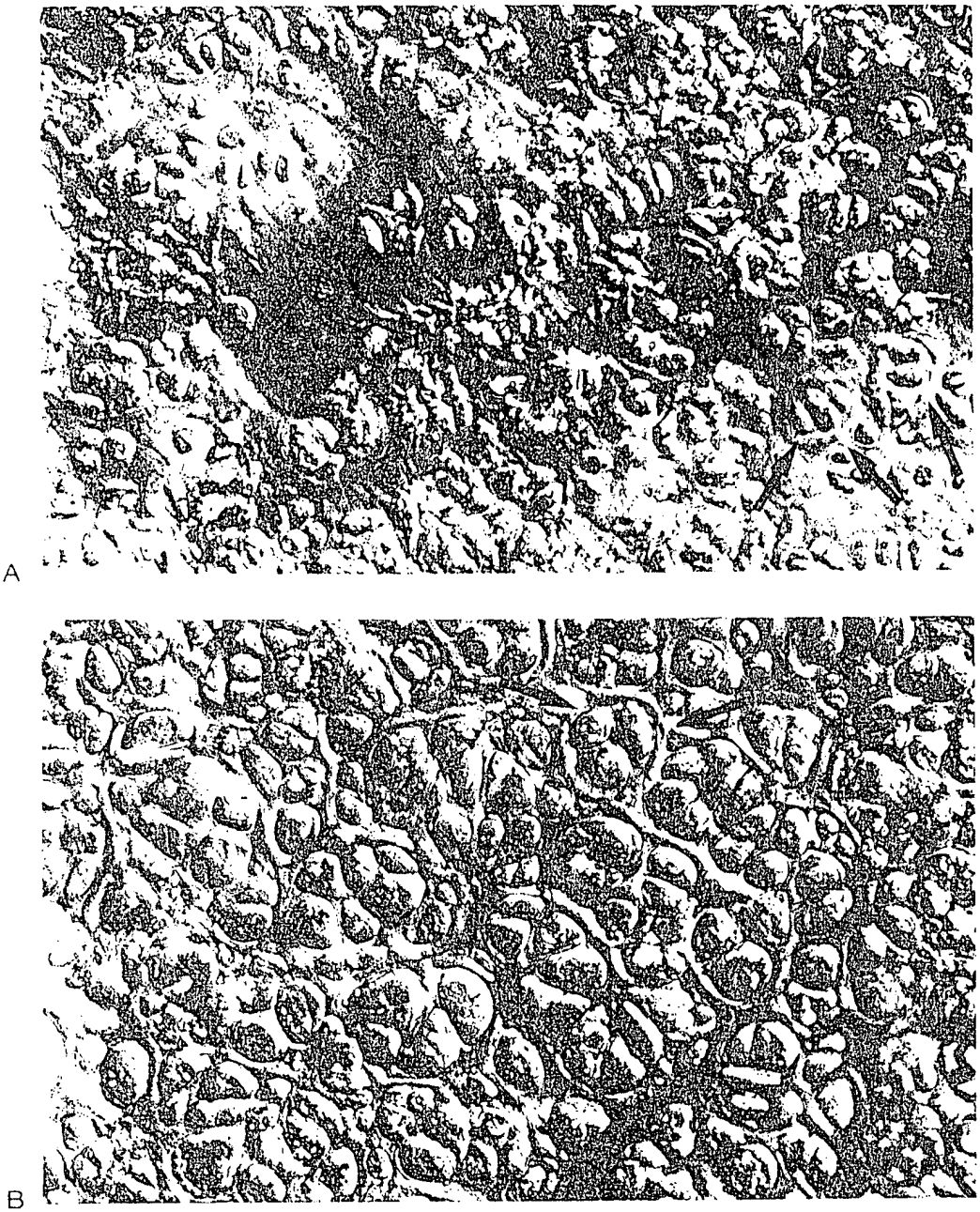

FIG. 3. Bovine articular chondrocytes cultured for 6 weeks in an agarose gel (Hoffman modulation contrast, ×225). **A:** Mixed population derived from middle-depth slices of cartilage; some chondrocytes are rounded and enclosed in matrix (*arrows*), whereas others lack visible matrix but have long cell processes (*arrowheads*). **B:** Chondrocytes isolated from deep slices of cartilage are more homogeneous; note the preponderence of groups of large, rounded hypertrophic cells encircled by dense matrix (*arrows*).

teristic of chondrocyte hypertrophy (e.g., synthesis of alkaline phosphatase) were also observed in porcine chondrocytes derived from deep cartilage and cultured as a monolayer (6). Human chondrocytes in liquid suspension also initiated synthesis of alkaline phosphatase, but cells from both the superficial and deep regions of the cartilage showed similar biochemical changes characteristic of maturation, although apparently without accompanying morphological changes

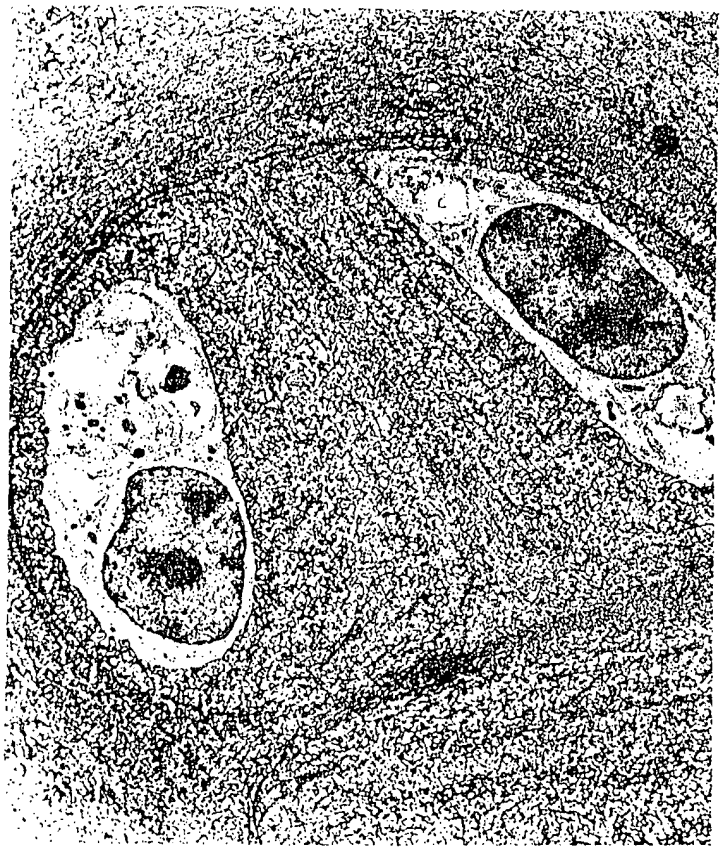

FIG. 4. Electron micrograph of cultured bovine chondrocytes derived from the radial zone of articular cartilage (×3160). These cells were cultured for 9 weeks in an agarose gel, and then they were fixed in the presence of ruthenium hexamine trichloride to stain proteoglycans. Note the extensive, well-organized matrix produced *in vitro*, with a pericellular region rich in proteoglycans; also note the collagen fibrils in the territorial matrix oriented predominantly parallel to the cell surface. (Electron microscopy courtesy of J. R. Kuszak.)

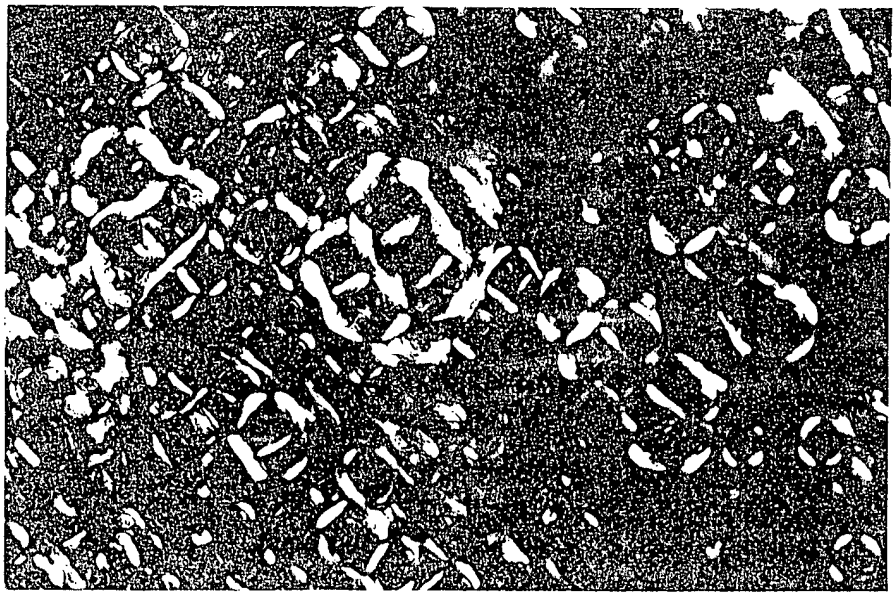

FIG. 5. Bovine articular chondrocytes derived from the entire thickness of cartilage. These cells were cultured for 11 weeks within agarose, and then they were viewed by polarizing microscopy (×75). Note the strong birefringence of the matrix partitions surrounding groups of cells.

(15). The calcium content of the medium was low, and the matrix did not become mineralized in these cultures (15).

CARTILAGE ORGAN CULTURES

The metabolism of different groups of chondrocytes has also been studied in organ cultures with the cells retained in their original matrix (8,9). When initially placed in culture, or after maintenance in medium lacking serum or other growth factors, the superficial zone showed the lowest rate of proteoglycan synthesis based upon the wet weight of the tissue, even though this region was the most cellular (8,9). However, proteoglycan synthesis was stimulated by serum growth factors, and interestingly, in organ cultures, the chondrocytes of the superficial zone showed the greatest response (8). It seems unlikely that the rate of diffusion of growth factors through the tissue was a limiting factor, because the results were similar whether the cartilage was (a) cultured as full-depth plugs and subdivided later for analysis or (b) cultured as thin slices separated according to distance from the articular surface (8). By contrast with serum, insulin (which can readily permeate cartilage matrix) stimulated proteoglycan synthesis to a similar degree in all zones of the tissue (8). The variation in response of chondrocytes to serum may have resulted from a differential partition of high-molecular-weight growth factors, which could readily penetrate the superficial zone but would have only limited access to cells in the middle and deep zones of cartilage which lie within a matrix rich in proteoglycans (8).

CONCLUSIONS AND FURTHER CHALLENGES

Subpopulations of chondrocytes isolated from their native matrix in superficial and deep regions of articular cartilage and cultured either as suspensions or as high-density monolayers show both morphological and quantitative metabolic differences which persist over a period of many weeks. These results are consistent with the retention and continued expression in culture of some intrinsic cellular differences. During their isolation from cartilage, the chondrocytes are completely denuded of their specialized matrix, and they are then placed under identical conditions of culture. However, they cannot be retained for a long period in the same milieu, because the chondrocytes themselves immediately begin to modify their microenvironments by elaborating a new extracellular matrix, and this in turn influences their metabolism. While subpopulations of chondrocytes from both superficial and deep zones continue to synthesize predominantly type II collagen and aggrecan in agarose gels, only the chondrocytes from deep in the tissue reestablish a highly organized matrix which closely resembles that in the deep zone of normal articular cartilage (4). The chondrocytes from the tangential zone of cartilage are normally enclosed within a dense matrix of fine collagen fibrils which has a relatively low concentration of proteoglycans, but these cells do not elaborate such a matrix under a variety of culture conditions so far tested.

Suspension within an agarose gel helps to preserve a spherical cell-shape which is important for normal phenotypic expression of chondrocytes, but even in this environment some cartilage cells eventually assume highly irregular contours with numerous processes. The zone I chondrocytes, in particular, are much more labile in their morphology than are deep cells, and they have a strong tendency to form monolayers and networks by spreading and migrating on any surfaces available within or around the agarose gel. Culture of chondrocytes suspended in liquid medium, and the absence of available surfaces for cellular attachment (apart from other chondrocytes), apparently resulted in better phenotypic stability of zone I chondrocytes (7). Nevertheless, in the culture environment, qualitative anabolic differences between populations from superficial and deep zones of human cartilage gradually diminished. Cells from both groups underwent an accelerated maturation and started to synthesize type X collagen and alkaline phosphatase (15). Thus, the conditions of culture which will induce cultured zone I chondrocytes to elaborate a matrix similar to that in the tangential zone of articular cartilage have not yet been realized. Furthermore, results of these experiments suggest that the heterogeneity of chondrocytes within articular cartilage stems to some degree from intrinsic cellular variation, but also results from differing environmental influences.

Within the joint, chondrocytes in the superficial zone are flattened and disc-shaped, oriented parallel to the articular surface. Perhaps this cell shape results partly from the forces of tension and compression in this part of the cartilage during development and growth of the epiphysis, and during normal loading with movement of the joint. Such a shape may also be a prerequisite for the normal phenotypic expression of superficial chondrocytes. It will be interesting to examine the morphology and metabolism of superficial chondrocytes, and to examine their capacity for reconstructing their characteristic matrix when cultured under tension, pressure, or other conditions which force the cells to retain a discoid shape. Such studies are planned, but the work is currently hampered by continuing difficulties in separating chondrocytes from the superficial zone in sufficient numbers, and with the least possible contamination with cells from deeper layers. If specific membrane markers for different groups of chondrocytes could be identified and prepared, these would facilitate more efficient cell separations.

The superficial zone of articular cartilage plays a vital role in the healthy joint by virtue of its unique location, structure, and material properties. It provides a smooth surface with low friction which withstands high tensile stresses, and it distributes the load over the surface of the joint, thereby protecting the underlying cartilage. However, the tangential layer is also the first region to show signs of deterioration in osteoarthritis; loss of integrity in this protective "skin" exposes the deeper cartilage to damaging stresses of loading during joint movement. Therefore, further attempts must be made to understand better the unique features of the superficial zone chondrocytes, to determine how their metabolism can be modulated to promote both the synthesis of appropriate macromolecules and the incorporation of these components into a strong, well-organized matrix.

If this goal could be attained for cultured chondrocytes, there would be better possibilities for understanding how the metabolism of these special cells could be manipulated within the intact joint in ways to maintain tissue integrity.

ACKNOWLEDGMENTS

We are grateful for support (for the preparation of this manuscript and for some of the studies described herein) from an Arthritis Research Grant of Werk Kalle-Albert of Hoechst Aktiengesellschaft (Germany) and from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIH grant 1-P50 A-39239). We sincerely appreciate the expert technical assistance of Robert R. Greenhill and David H. Pearl.

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DISCUSSION

Werb: What are the phenotypic differences between cells derived from the different zones?

Aydelotte: The cells from the superficial zone degrade proteoglycans more rapidly. That's the only way they seem to be more metabolically active than the cells from the deep zone; otherwise they synthesize less proteoglycans and less collagen.

Werb: But have they become more fibroblastic? For example, do they make type I collagen?

Aydelotte: I think they make some type I collagen when they flatten and attach to a surface. It would also be interesting to know if they make type IIA collagen rather than type IIB, because Linda Sandell stated that cells that make type IIA collagen do not accumulate matrix.

Kimura: We have a cell line from a human chondrosarcoma which morphologically exhibits many of the features that you see for these superficial cells, including cell processes. They make a large cartilage-type proteoglycan that has keratan sulfate on it. They stain positively with an antibody against type II collagen. Therefore, I am not sure one can correlate cell morphology with biochemical expression.

von der Mark: Do the chondrocyte–agarose cultures synthesize any type IX collagen? Furthermore, was there any type X synthesized in your long-term cultures?

Aydelotte: We have shown by immunohistochemistry that both type IX and type XI collagen are made. Their distribution is similar to that of type II collagen. SDS-PAGE analyses in a calcification study show that the cells from deep cartilage synthesize type X collagen, but not in great quantities.

Campion: Danger to cartilage integrity is thought to come from the synovial cavity, and it seems paradoxical that the superficial cells seem to be more responsive to cytokines such as IL-1, which are implicated in some joint diseases. Are we looking the wrong way round? Is the danger to cartilage coming from the bone side?

Aydelotte: No, not really! I think cells near the articular surface have special roles such as in controlling what passes through that zone to the deeper layers. I think some of Derek Cooke's work on deposition of immune complexes may illustrate this specialized function.